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CELL, vol. 40, January 1985, pages 9-17, MIT; S. WAIN-HOBSON et al.: "Nucleotide sequence of the AIDS virus, LAV"

SCIENCE, vol. 229, 23rd August 1985, pages 759-762; F. WONG-STAAL et al.: "Genomic diversity of human T-lymphotropic virus type III (HTLV-III)"

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THE LANCET, 23rd June 1984, pages 1383-1385; A. ELLRODT et al.: "Isolation of human T-lymphotropic retrovirus(LAV) from Zairian married couple, one with aids, one with prodromes"

Description

The present invention relates to viruses capable of inducing lymphadenopathies (denoted below by the abbreviation LAS) acquired immunodepressive syndromes (denoted below by the abbreviation AIDS), to antigens of said viruses, particularly in a purified form, and to processes for producing these antigens, particularly antigens of the envelopes of these viruses. The invention also relates to polypeptides, whether glycosylated or not, encoded by said DNA sequences.

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The invention also relates to cloned DNA sequences hybridizable to genomic RNA and DNA of the new lymphadenopathy associated viruses (LAV) disclosed hereafter, to processes for their preparation and their uses. It relates more particularly to stable probes including a DNA sequence which can be used for the detection of the new LAV viruses or related viruses or DNA proviruses in any medium, particularly biological, samples, containing of any them.

An important genetic polymorphism has been recognized for the human retrovirus at the origin of the acquired immune deficiency syndrome (AIDS) and other diseases, like lymphadenopathy syndrome (LAS), AIDS-related complex (ARC) and probably some encephalopathies (for review see Weiss, 1984). Indeed all of the isolates analyzed until now have a distinct restriction map, even if recovered from the same place and time (BENN et al., 1985). Identical restriction maps have only been observed for the first two isolates designated lymphadenopathy-associated virus, LAV (ALIZON et al., 1984) and human T-cell lymphotropic virus type 3, HTLV-3 (HAHN et al., 1984) and thus appears as an exception. The genetic polymorphism of the AIDS virus was better assessed after the determination of the complete nucleotide sequence of LAV (WAIN-HOBSON et al., 1985), HTLV-3 (RATNER et al., 1985; MUESING et al., 1985) and of a third isolate designated AIDS-associated retrovirus, ARV (SANCHEZ-PESCADOR et al., 1985). In particular it appeared that, besides the nucleic acid variations responsible for the restriction map polymorphism, isolates could differ significantly at the protein level, especially in the envelope (up to 13 % of difference between ARV and LAV), by both amino-acids substitutions and reciprocal insertions-deletions (RABSON and MARTIN, 1985).

Nevertheless the differences mentioned above do not go as far as to destroy a level of immunological relationship sufficient, as evidenced by the capabilities of similar proteins, i. e. core proteins of similar nature, such as the p25 proteins, or of similar envelope glycoproteins, such as the 110-120 kD glycoproteins, to immunologically cross-

react. Accordingly the proteins of any of said LAV viruses can be used for the in vitro detection of antibodies induced in vivo and present in biological fluids obtained from individuals infected with the other LAV variants. Therefore these viruses are grouped in a class of LAV viruses, hereafter generally said to belong to the class of LAV-1 viruses.

The invention stems from the discovery of new viruses which although held as responsible of diseases which are clinically related to AIDS and still belonging to the class of "LAV-1 viruses", differ genetically to a much larger extent from the above mentioned LAV variants.

The new viruses are basically characterized by the DNA sequences which are shown in Figures 7A to 7J (LAV_{ELI}) and figures 8A to 8I (LAVM_{AL}) respectively.

The invention further relates to variants of the new viruses the RNAs of which or the related cDNAs derived from said RNAs are hybridizable to corresponding parts of the cDNAs of either LAV_{ELI} or LAV_{MAL}.

The invention also relates to the DNAs themselves of said viruses, hybridizable with the genomic RNA of either LAV_{ELI} or LAV_{MAL}. Particularly said DNAs consist of said cDNAs or of recombinant DNAs containing said cDNAs.

It further relates to DNA recombinants containing DNAs of either LAV_{ELI} or LAV_{MAL} or of related viruses. It is of course understood that DNAs which would include some deletions or mutations which would not substantially alter their capability of also hybridizing with the retroviral genomes of LAV_{ELI} or LAV_{MAL} are to be considered as forming obvious equivalents of the DNAs more specifically referred to hereabove.

The invention also relates more specifically to cloned probes which can be made starting from any DNAs according to the invention, thus to recombinant DNAs containing such DNAs, particularly any plasmids amplifiable in procaryotic or eucaryotic cells and carrying said DNAs.

Using the cloned DNA containing a DNA of LAV_{ELI} or of LAV_{MAL} as a molecular hybridization probe - either by marking with radionucleotides or with fluorescent reagents - LAV virion RNA may be detected directly e.g. in the blood, body fluids and blood products (e.g. of the antihemophilic factors such as Factor VIII concentrates). A suitable method for achieving that detection comprises immobilizing virus onto a support e.g. nitrocellulose filters, etc., disrupting the virion and hybridizing with labelled (radiolabelled or "cold" fluorescent- or enzyme-labelled) probes. Such an approach has already been developed for Hepatitis B virus in peripheral blood (according to SCOTTO J. et al. Hepatology (1983), 3, 379-384).

Probes according to the invention can also be

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used for rapid screening of genomic DNA derived from the tissue of patients with LAV related symptoms, to see if the proviral DNA or RNA present in host tissue and other tissues are related to LAV_{ELI} or LAV_{MAL}.

A method which can be used for such screening comprise the following steps: extraction of DNA from tissue, restriction enzyme cleavage of said DNA, electrophoresis of the fragments and Southern blotting of genomic DNA from tissues, subsequent hybridization with labelled cloned LAV provival DNA. Hybridization in situ can also be used

Lymphatic fluids and tissues and other nonlymphatic tissues of humans, primates and other mammalian species can also be screened to see if other evolutionnary related retrovirus exist. The methods referred to hereabove can be used, although hybridization and washings would be done under non stringent conditions.

The DNA according to the invention can be used also for achieving the expression of LAV viral antigens for diagnostic purposes as well as far the production of a vaccine against LAV. Fragments of particular advantage in that respect will be discussed later.

The methods which can be used are multifold:
a) DNA can be transfected into mammalian cells with appropriate selection markers by a variety of techniques, calcium phosphate precipitation, polyethylene glycol, protoplast-fusion, etc...

- b) DNA fragments corresponding to genes can be cloned into expression vectors for <u>E. coli</u>, yeast- or mammalian cells and the resultant proteins purified.
- c) The provival DNA can be "shot-gunned" (fragmented) into procaryotic expression vectors to generate fusion polypeptides. Recombinant producting antigenically competent fusion proteins can be identified by simply screening the recombinants with antibodies against LAV_{ELI} or LAV_{MAL} antigens.

Particular reference in that respect is made to those portions of the genomas of LAV_{EU} and LAV_{MAL} which, in the drawings, are shown to belong to open reading frames and which encode the products having the polypeptidic backbones shown.

More particularly, the invention relates to the different polypeptides which appear in figures 7A to 8I. Methods disclosed in European application 0 178 978 and in PCT application PCT/EP 85/00548 filed on Oct.18, 1985 are applicable for the production of such peptides from the corresponding viruses.

The present invention further aims at providing polypeptides containing sequences in common with polypeptides comprising antigenic determi-

nants included in the proteins encoded and expressed by the LAV_{ELI} or of LAV_{MAL} genome. An additional object of the invention is to further provide means for the detection of proteins related to the LAV viruses, particularly for the diagnosis of AIDS or pre-AIDS or, to the contrary, for the detection of antibodies against the LAV virus or proteins related therewith, particularly in patients afflicted with AIDS or pre-AIDS or more generally in asymtomatic carriers and in blood-related products. Finally the invention also aims at providing immunogenic polypeptides, and more particularly protective polypeptides for use in the preparation of vaccine compositions against AIDS or related syndroms.

The invention relates also to polypeptide fragments having lower molecular weights and having peptide sequences or fragments in common with those shown in figures 7A to 8I. Fragments of smaller sizes may be obtained by resorting to known techniques. For instance such a method comprises cleaving the original larger polypeptide by enzymes capable of cleaving it at specific sites. By way of examples of such proteins, may be mentioned the enzyme of Staphylococcyus aureus V8, a-chymotrypsine, "mouse sub-maxillary gland protease" marketed by the BEOHRINGER company, Vibrio alginolyticus chemovar iophagus collagenase, which specifically recognizes said peptides Gly-Pro and Gly-Ala, etc.

Other features of this invention will appear in the following disclosure of the data obtained starting from LAV_{ELI} and LAV_{MAL}, in relation to the drawings in which:

- Figs 1A and 1B provide restriction maps of the genomas of LAV_{EU} and LAV_{MAL} as compared to LAV_{BRU} (a known LAV isolate deposited at CNCM under number I-232 on July 15th, 1983);
- Fig. 2 shows the comparative maps setting forth the relative positions of the open reading frames of the above genomas;
- Figs. 3A-3F (sometimes also designated globally hereafter by fig. 3) indicate the relative correspondance between the proteins (or glycoproteins) encoded by the open reading frames, whereby aminoacid residues of protein sequences of LAVELI and LAVMAL are in vertical alinment with corresponding aminoacid residues (numbered) of corresponding or homologous proteins glycoproteins of LAVBRU;
- Figs. 4A-4B (sometimes also designated globally hereafter by fig. 4) provide for quantitation of the sequence divergence between homologous proteins of LAV_{BRU}, LAV_{ELI} and LAV_{MAL};
- Fig. 5 shows diagrammatically the degree of

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- divergence of the different virus enveloppe proteins;
- Figs. 6A and 6B (or Fig. 6 when viewed altogether) render apparent the direct repeats which appear in the proteins of the different AIDS virus isolates.
- Figs. 7A-7J and 8A-8I show the full nucleotidic sequences of LAV_{ELI} and LAV_{MAL} respectively.

RESULTS

Characterization and molecular cloning of two African isolates.

The different AIDS virus isolates concerned are designated by three letters of the patients name, LAV_{BRU} refeRring to the prototype AIDS virus isolated in 1983 from a French homosexual patient with LAS and thought to have been infected in USA in the preceding years (Barré-Sinoussi et al., 1983). Both of the African patients originated from Zaire; LAV_{ELI} was recovered in 1983 from a 24 year old woman with AIDS, and LAV_{MAL} in 1985 from a 7 year old boy with ARC, probably infected in 1981 after a blood-transfusion in Zaire, since his parents were LAV-seronegative.

Recovery and purification of each of the two viruses were performed according to the method disclosed in European Patent Application 84 401834/138 667 filed on September 9, 1984.

LAV_{ELI} and LAV_{MAL} are indistinguishable from the previously characterized isolates by their structural and biological properties in vitro. Virus metabolic labelling and immune precipitation by patients ELI and MAL sera, as well as reference sera, showed that the proteins of LAV_{ELI} and LAV_{MAL} had the same molecular weight (MW) and cross-reacted immunologically with those of prototype AIDS virus (data not shown) of the "LAV 1" class.

Reference is again made to European Application 178 978 and International Application PCT/EP 85/00548 as concerns the purification, mapping and sequencing procedures used herein. See also "experimental procedures" and "legends of the figures" hereafter.

Primary restriction enzyme analysis of LAV_{ELI} and LAV_{MAL} genomes was done by southern blot with total DNA derived from acutely infected lymphocytes, using cloned LAV_{BRU} complete genome as probe. Overall cross-hybridization was observed under stringent conditions, but the restriction profiles of the Zairian isolates were clearly different. Phage lambda clones carrying the complete viral genetic information were obtained and further characterized by restriction mapping and nucleotide sequence analysis; clone E-H12 is derived from LAV_{ELI} infected cells and contains an integrated provirus with 5' flanking cellular sequences but a

truncated 3' long terminal repeat (LTR); clone M-H 11 was obtained by complete HindIII restriction of DNA from LAV_{MAL}-infected cells, taking avantage of the existence of a unique HindIII site in the LTR. M-H 11 is thus probably derived from unintegrated viral DNA since that species was at least ten times more abundant than integrated provirus.

Figure 1B gives a comparaison of the restriction maps of LAV_{ELI}, LAV_{MAL} and prototype LAV_{BRU}. all three being derived from their nucleotide sequences, as well of three Zairian isolates previously mapped for seven restriction enzymes (Benn et al., 1985). Despite this limited number, all of the profiles are clearly different (out of the 23 sites making up the map of LAV^{BRU} only seven are present in all six maps presented), confirming the genetic polymorphism of the AIDS virus. No obvious relationship is apparent between the five Zairian maps, and all of their common sites are also found in LAV_{BRU}.

Conservation of the genetic organization.

The genetic organization of LAV_{ELI} and LAV_{MAL} as deduced from the complete nucleotide sequences of their cloned genomes is identical to that found in other isolates, i.e. 5'-gag-pol-central region-env-F3'. Most noticeable is the conservation of the "central region" (fig. 2), located between the pol and env genes, which is composed of a series of overlapping open reading frames (orf) we had previously designated Q, R, S, T, and U after observing a similar organization in the ovine lentivirus visna (Sonigo et al., 1985). The product of orf S (also designated "tat") is implicated in the transactivation of virus expression (Sodroski et al., 1985; Arya et al., 1985); the biological role of the product of orf Q (also designated "sor" or orf A) is still unknown (Lee et al., 1986; kang et al., 1986). Of the three other orfs (R, T, and U), only orf R is likely to be a seventh viral gene, for the following reasons : the exact conservation of its relative position with respect to Q and S (fig. 2), the constant presence of a possible splice acceptor and of a consensus AUG initiator codon, its similar codon usage with respect to viral genes, and finally the fact that the variation of its protein sequence within the different isolates is comparable to that of gag, pol and Q (see Fig. 4).

Also conserved are the sizes of the U3, R and U5 elements of the LTR (data not shown), the location and sequence of their regulatory elements such as TATA box and AATAAA polyadenylation signal, and their flanking sequences i.e. primer binding site (PBS) complementary to 3' end of

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tRNA^{LYS}

and polypurine tract (PPT). Most of the genetic variability within the LTR is located in the 5' half of U3 (which encodes a part of orf F) while the 3' end of U3 and R, which carry most of the cis-acting regulatory elements: promoter, enhancer and trans-activating factor receptor (Rosen et al., 1985), as well as the U5 element are well-conserved.

Overall, it clearly appears that the Zairian isolates belong to the same type of retrovirus as the previously sequenced isolates of American or European origin.

Variability of the viral proteins.

Despite their identical genetic organization, these isolates show substantial differences in the primary structure of their proteins. The amino acid sequences of LAV_{ELI} and LAV_{MAL} proteins are presented in figures 3A-3F (to be examined in conjunction with Figs. 7A-7J and 8A-8I), aligned with those of LAV_{BRU} and ARV 2. Their divergence was quantified as the percentage of amino-acids substitutions in two-by-two alignments (Fig. 4). We have also scored the number of insertions and deletions that had to be introduced in each of these alignments.

Three general observations can be made. First, the protein sequences of the African isolates are more divergent from LAVBRU than are those of HTLV-3 and ARV 2 (Fig. 4A); similar results are obtained if ARV 2 is taken as reference (not shown). The range of genetic polymorphism between isolates of the AIDS virus is considerably greater than previously observed. Second, our two sequences confirm that the envelope is more variable than the gag and pol genes. Here again, the relatively small difference observed between the env of LAVBRU and HTLV-3 appears as an exception. Third, the mutual divergence of the two African isolates (Fig. 4B) is comparable to that between LAVBRU and either of them; as far as we can extrapolate from only three sequenced isolates from the USA and Europe and two from Africa, this is indicative of a wider evolution of the AIDS virus in Africa.

gag and pol: Their greater degree of conservation compared to the envelope is consistent with their encoding important structural or enzymatic activities. Of the three mature gag proteins, the p25 which was the first recognized immunogenic protein of LAV (Barré-Sinoussi et al., 1983) is also the better conserved (fig. 3). In gag and pol, differences between isolates are principally due to point mutations, and only a small number of insertional

or deletional events is observed. Among these, we must note the presence in the over-lapping part of gag and pol of LAV_{BRU} of an insertion of 12 aminoacids (AA) which is encoded by the second copy of a 36 bp direct repeat present only in this isolate and in HTLV-3. This duplication was omitted because of a computing error in the published sequence of LAV_{BRU} (position 1712, Wain-Hobson et al., 1985) but was indeed present in the HTLV-3 sequences (Ratner et al., 1985; Muesing et al., 1985).

env: Three segments can be distinguished in the envelope glycoprotein precursor (Allan et al., 1985; Montagnier et al., 1985; DiMarzoVeronese et al., 1985). The first is the signal peptide (positions 1-33 in fig. 3), and its sequence appears as variable; the second segment (pos. 34-530) forms the outer membrane protein (OMP or gp110) and carries most of the genetic variations, and in particular almost all of the numerous reciprocal insertions and deletions; the third segment (531-877) is separated from the OMP by a potential cleavage site following a constant basic stretch (Arg-Glu-Lys-Arg) and forms the transmembrane protein (TMP or gp 41) responsible for the anchorage of the envelope glycoprotein in the cellular membrane. A better conservation of the TMP than the OMP has also been observed between the different murine leukemia viruses (MLV, Koch et al., 1983), and could be due to structural constraints.

From the alignment of figure 3 and the graphical representation of the envelope variability shown in figure 5, we clearly see the existence of conserved domains, with little or no genetic variation, and hypervariable domains, in which even the alignment of the different sequences is very difficult, because of the existence of a large number of mutations and of reciprocal insertions and deletions. We have not included the sequence of the envelope of the HTLV-3 isolate since it so close to that of LAVBRU (cf. fig. 4), even in the hypervariable domains, that it did not add anything to the analysis. While this graphical representation will be refined by more sequence data, the general profile is already apparent, with three hypervariable domains (Hyl, 2 and 3) all being located in the OMP, and separated by three well-conserved stretches (residues 37-130, 211-289, and 488-530 of fig. 3 alignment) probably associated with important biological functions.

In spite of the extreme genetic variability, the folding pattern of the envelope glycoprotein is probably constant. Indeed the position of virtually all of the cysteine residues is conserved within the different isolates (fig. 3 and 5), and the only three variable cysteines fall either in the signal peptide or in the very C-terminal part of the TMP. The hypervariable domains of the OMP are bounded by

conserved cysteines, suggesting that they may represent loops attached to the common folding pattern. Also the calculated hydropathic profiles (Kyte and Doolittle, 1982) of the different envelope proteins are remarkably conserved (not shown).

About half of the potential N-glycosylation sites, Asn-X-Ser/Thr, found in the envelopes of the Zairian isolates map to the same positions in LAV_{BRU} (17/26 for LAV_{ELI} and 17/28 for LAV_{MAL}). The other sites appear to fall within variable domains of env, suggesting the existence of differences in the extent of envelope glycosylation between different isolates.

Other viral proteins: Of the three other identified viral proteins, the p27 encoded by orf F, 3' of env (Allan et al., 1985b) is the most variable (fig. 4). The proteins encoded by orfs Q and S of the central region are remarkable by their absence of insertions/deletions. Surprisingly, a high frequency of aminoacids substitutions, comparable to that observed in env, is found for the product of orf S (trans-activating factor). On the other hand, the protein encoded by orf Q is no more variable than gag. Also noticeable is the lower variation of the proteins encoded by the central regions of LAV_{ELI} and LAV_{MAL}.

DISCUSSION

With the availability of the complete nucleotide sequence from five independent isolates, some general features of the AIDS virus genetic variability are now emerging. Firstly, its principal cause are point mutations very often resulting in aminoacid substitutions, and which are more frequence in the 3' part of the genome (orf S, env and orf F). Like all RNA viruses, the retroviruses are thought to be highly subject to mutations caused by errors of the RNA polymerases during their replication, since there is no proofreading, of this step (Holland et al., 1982; Steinhauer and Holland, 1986).

Another source of genetic diversity are insertions/deletions. From the figure 3 alignments, insertional events seem to be implicated in most of the cases, since otherwise deletions should have occurred in independant isolates at the precisely the same location. Furthermore, upon analyzing these insertions, we have observed that they most often represent one of the two copies of a direct repeat (fig. 6). Some are perfectly conserved like the 36 bp repeat in the gag-pol overlap of LAVBRU -(fig. 6-a); others carry point mutations resulting in aminoacid substitutions, and as a consequence, they are more difficult to observe, though clearly present, in the hypervariable domains of env (cf. fig. 6-g and -h). As noted for point mutations, env gene and orf F also appear as more susceptible to that form of genetic ariation than the rest of the

genome. The degree of conservation of these repeats must be related to their date of occurrence in the analyzed sequences: the more degenerated, the more ancient. A very recent divergence of LAV_{BRU} and HTLV3 is suggested by with extremely low number of mismatched AA between their homologous proteins. However, one of the LAVBRU repeats (located in the Hyl domain of env, fig. 6-f) is not present in HTLV3, indicating that this generation of tandem repeats is a rapid source of genetic diversity. We have found no traces of such a phenomenon, even when comparing very closely related viruses, such as the Mason-Pfizer monkey virus, MPMV (Sonigo et al., 1986), and an immunosuppressive simian virus, SRV-1 (Power et al., 1986). Insertion or deletion of one copy of a direct repeat have been occasionally reported in mutant retroviruses (Shimotohno and Temin, 1981: Darlix, 1986), but the extent at which we observe this phenomenon is unprecedented.

The molecular basis of these duplications is unclear, but could be the "copy-choice" phenomenon, resulting from the diploidy of the retroviral genome (Varmus and Swanstrom, 1984; Clark and Mak, 1983). During the synthesis of the first-strand of the viral DNA, jumps are known to occur from one RNA molecule to another, especially when a break or a stable secondary structure is present on the template; an inaccurate re-initiation on the other RNA template could result in the generation (or the elimination) of a short direct repeat.

Genetic variability, and subsequent antigenic modifications, have often been developed by microorganisms as a means to escape the host's immune response, either by modifying their epitopes during the course of the infection, as in trypanosomes (Borst and Cross, 1982), or by generating a large repertoire of antigens, as observed in influenza virus (Webster et al., 1982), As the human AIDS virus is related to animal lentiviruses (Sonigo et al., 1985; Chiu et al., 1985), its genetic variability could be a source of antigenic variation, as can be observed during the course of the infection by the ovine lentivirus visna (Scott et al., 1979 ; Clements et al., 1980) or by the equine infectious anemia virus (EIAV, Montelaro et al., 1984). However, a major discrepancy with these animal models is the extremely low, if any, neutralizing activity of the sera of individuals infected by the AIDS virus, whether they are healthy carriers, displaying minor symptoms or afflicted with AIDS (Weiss et al., 1985; Clavel, et al., 1985). Furthermore, even for the visna virus the exact role of antigenic variation in the pathogenesis is unclear (Thormar et al., 1983; Lutley et al., 1983). We rather feel that genetic variation represents a general selective advantage for lentiviruses by allowing an adaptation to different environments, for exam-

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ple by modifying their tissue or host tropisms. In the particular case of the AIDS virus, rapid genetic variations are tolerated, especially in the envelope; they could allow the virus to get adapted to different "micro-environments" of the membrane of their principal target cells, namely the T4 lymphocytes. These "micro-environments" could result from the immediate vicinity of the virus receptor to polymorphic surface proteins, differring either between individuals or between clones of lymphocytes.

Conserved domains in the AIDS virus envelope.

Since the proteins of most of the isolates are antigenically cross-reactive, the genotypic differences do not seem to affect the sensitivity of actual diagnostic tests, based upon the detection of antibodies to the AIDS virus and using purified virions as antigens. They nevertheless have to be considered for the development of the "second-generation" tests, that are expected to be more specific. and will use smaller synthetic or genetically-engineered viral antigens. The identification of conserved domains in the highly immunogenic envelope glycoprotein, and also the core structural proteins (gag), is very important for these tests. The conserved stretch found at the end of the OMP and the beginning of the TMP (490-620, fig. 3) could be a good candidate, since a bacterial fusion protein containing this domain was welldetected by AIDS patients sera (Chang et al., 1985).

The envelope, specifically the OMP, mediates the interaction between a retrovirus and its specific cellular receptor (DeLarco and Todaro, 1976; Robinson et al., 1980). In the case of the AIDS virus, in vitro binding assays have shown the interaction of the envelope glycoprotein gp110 with the T4 cellular surface antigen (McDougal et al., 1986), already thought to be, or closely associated to, the virus receptor (Klatzmann et al., 1984; Dagleish et al., 1984). Identification of the AIDS virus envelope domains that are responsible for this interaction (receptor-binding domains) appears as fundamental for understanding of the host-viral interactions, but also for designing a protective vaccine, since an immune response against these epitopes could possibly elicit neutralizing antibodies. As the AIDS virus receptor is at least partly formed of a constant structure, the T4 antigen, the binding site ofthe envelope-is unlikely to be exclusively encoded by domains undergoing drastic genetic changes between isolates, even if these could be implicated in some kind of an "adaptation". One, or several of the conserved domains of the OMP (residues 37-130, 211-289, and 488-530 of fig. 3 alignment) brought together by the folding of the protein, must

play a part in the virus-receptor interaction, and this can be explored with synthetic or genetically-engineered peptides derived from these domains, either by direct binding assays, or indirectly by assaying the neutralizing activity of specific antibodies raised against them.

African AIDS viruses

Zaire and the neighbouring countries of Central Africa are considered as an area of endemic for the AIDS virus infection, and the possibility that the virus has emerged in Africa has became a subject of intense controversy (see Norman, 1985). From the present study, it is clear that the genetic organization of Zairian isolates is the same as that of american isolates, thereby indicating a common origin. The very important sequence differences observed between the proteins are consistent with a divergent evolutionary process. In addition, the two African isolates are mutually more divergent than the American isolates already analyzed; as far as that observation can be extrapolated, it suggests a longer evolution of the virus in Africa, and is also consistent with the fact that a larger fraction of the population is exposed than in developed countries.

A novel human retrovirus with morphology and biological properties (cytopathogenicity, T4 tropism) similar to those of LAV, but nevertheless clearly genetically and antigenically distinct from that latter, was recently isolated from two patients with AIDS originating from Guinea Bissau, West-Africa (Clavel et al., 1986). In the neighbouring Senegal the population seems exposed to a retrovirus also distinct from LAV, but apparently non pathogenic (Barin et al., 1985; Kanki et al., 1986). Both of these novel African retroviruses seem to be antigenically related to the simian T-cell lymphotropic virus, STLV-III, shown to be widely present in healthy African green monkeys and other simian species (Kanki et al. 1985). This raises the possibility of a large group of African primate lentiviruses, ranging from the apparently non-pathogenic simian viruses to the LAV-type viruses. Their precise relationship will only be known after their complete genetic characterization, but it is already very likely that they have evolved from a common progenitor. The important genetic variability we have observed between isolates of the AIDS virus in Central Africa is probably a hallmark of this entire group, and may account for the apparently important genetic divergence between its members (loss of cross-antigenicity in the envelopes). In this sense the conservation of the tropism for the T4 lymphocytes suggests that it is a major advantage acquired by these retroviruses.

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EXPERIMENTAL PROCEDURES

Virus isolations

LAV_{ELI} and LAV_{MAL} were isolated from the peripheral blood lymphocytes of the patients as described (Barré-Sinoussi et al., 1983); briefly, the lymphocytes were fractionated and co-cultivated with phytohaemagglutinin-stimulated normal human lymphocytes in the presence of interleukin 2 and anti-alpha interferon serum. Viral production was assessed by cell-free reverse transcriptase (RT) activity assay in the cultures and by electron microscopy.

Molecular cloning

Normal donor lymphocytes were acutely infected (104 cpm of RT activity/106 cells) as described (Barré-Sinoussi et al., 1983), and total DNA was extracted at the beginning of the RT activity peak. For LAV_{ELI}, a lambda library using the L47-1 vector (Loenen and Brammar, 1982) was constructed by partial HindIII digestion of the DNA as already described (Alizon et al., 1984). For LAVMAL, DNA from infected cells was digested to completion with HindIII and the 9-10kb fraction was selected on 0.8 % low melting point agarose gel and ligated into L47-1 HindIII arms. About 5.105 plaques for LAV_{ELI} and 2.10⁵ for LAV_{MAL}, obtained by in vitro packaging (Amersham) were plated on E. coli LA101 and screened in situ under stringent conditions, using the 9 kb Sacl insert of the clone lambda J19 (Alizon et al., 1984) carrying most of the LAV_{BRII} genome as probe. Clones displaying positive signals were plaque-purified and propagated on E. coli C600 recBC, and two recombinant phages carrying the complete genetic information of LAV_{ELI} (E-H12) and LAV_{MAL} (M-H11) were further characterized by restriction mapping.

Nucleotide sequence strategy

Viral fragments derived from E-H12 and M-H11 were sequenced by the dideoxy chain terminator procedure (Sanger et al., 1977) after "shotgun" cloning in the M13mp8 vector (Messing and Viera, 1982), as previously described (Sonigo et al., 1985). The viral genome of LAV_{ELI} is 9176 nucleotides, that of LAV_{MAL} 9229 nucleotides long. Each nucleotide was determined from more than 5 independent—clones—on—average.—Complete nucleotide sequences are not presented in this article for obvious reasons of space limitation but are freely available upon request to the authors, until they are released through sequence data banks.

LEGEND OF THE FIGURES

Figure 1: Restriction map analysis of AIDS virus isolates.

A/ Restriction maps of the inserts of phage lambda clones derived from cells infected with LAV_{ELI} (E-H12) and LAV_{MAL} (M-H11). The schematic genetic organization of the AIDS virus has been drawn above the maps. The LTRs are indicated by solid boxes. A:Aval-B:Bam HI-Bg:BgIII-E:EcoRI - H:HindIII - Hc:HincII - K:KpnI-N:Nde I-P:PstI-S:SacI-X:Xbal. Asterisks indicate the HindIII cloning sites in lambda L47-1 vector. B/Comparison of the sites for seven restriction enzymes in six isolates : the prototype AIDS virus LAVBRU, LAVMAL and LAVELI; Z1, Z2, Z3 are Zairian isolates with published restriction maps (Benn et al., 1985). Restriction sites are represented by the following symbols: Bglll; EcoRI; HincII; HindIII; KpnI; NdeI; SacI.

Figure 2: Conservation of the genetic organization of the central region in AIDS virus isolates.

Stop codons in each phse are represented as vertical bars. Vertical arrows indicate possible AUG initiation codons. Splice acceptor (A) and donor (D) sites identified in subgenomic viral mRNA (Muesing et al., 1985) are shown below the graphic of LAV_{BRU}, and corresponding sites in LAV_{ELI} and LAW_{MAL} are indicated. PPT indicates the repeat of the polypurine tract flanking the 3'LTR. As observed in LAV_{BRU} (Wain-Hobson et al., 1985), the PPT is repeated 256 nucleotides 5' to the end of the pol gene in both our sequences, but this repeat is degenerated at two positions in LAV_{ELI}.

Figure 3: Alignment of the protein sequences of four AIDS virus isolates.

Isolate LAVBRU (Wain-Hobson et al., 1985) is taken as reference; only differences with LAVBRU are noted for ARV2 (Sanchez-Pescador et al., 1985) and the two Zairian isolates LAV_{MAL} and LAV_{ELI}. A minimal number of gaps (-) was introduced in the alignments. The NH2-termini of p25gag and p1899 are indicated (Sanchez-Pescador, 1985). The potential cleavage sites in the envelope precursor (Allan et al., 1985a; diMarzo-Veronese, 1985) separating the signal peptide (SP), the outer membrane protein (OMP) and the transmembrane protein (TMP) are indicated as vertical arrows; conserved cysteines are indicated by black circles and variable cysteines are boxed. The one letter code for amino acids is: A:Ala; C:Cys; D:Asp; E:Glu ; F:Phe ; G:Gly ; H :His ; I:lle ; K:Lvs ; L:Leu ; M:Met; N:Asn; P:Pro; Q:Gln; R:Arg; S:Ser;

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T:Thr; V:Val; W:Trp; Y:Tyr.

Figure 4: Quantitation of the sequence divergence between homologous proteins of different isolates.

Part A of each table gives results deduced from two-by-two alignments using the proteins of LAV_{BRU} as reference, part B those of LAV_{ELI} as reference. Sources: Muesing et al., 1985 for HTLV-3; Sanchez-Pescador et al., 1985 for ARV 2 and Wain-Hobson et al., 1985 for LAVBRU. For each case of the tables, the size in amino-acids of the protein (calculated from the first methionine residue, or from the beginning of the orf for pol) is given at the upper left part. Below are given the number of deletions (left) and insertions (right) necessary for the alignment. The large numbers in bold face represent the percentage of amino-acids substitutions (insertions/deletions being excluded). Two by two alignments were done with computer assistance Wilburg and Lipman, 1983), using a gag penalty of 1, K-tuple of 1, and window of 20, except for the hypervariable domains of env, where the number of gaps was made minimum, and which are essentially aligned as shown in fig. 3. The sequence of the predicted protein encoded by orf R of HTLV-3 has not been compared because of a premature termination relative to all other isolates.

Figure 5: Variability of the AIDS virus envelope protein.

For each position x of the alignment of env - (Fig. 3), variability V(x) was calculated as

number of different amino-acids at position x V(x) = frequency of the most abundant amino-acid at position x.

Gaps in the alignments are considered as another amino-acid. For an alignment of 4 proteins, V(x) ranges from 1 (identical AA in the 4 sequences) to 16 (4 different AA). This type of representation has previously been used in a compilation of the AA sequence of immunoglobulins variable regions (Wu and Kabat, 1970). Vertical arrows indicate the cleavage sites; asterisks represent potential N-glysosylation sites (N-X-S/T) conserved in all four isolates; black triangles represent conserved cysteine residues. Black lozanges mark the three major hydrophobic domains. OMP: outermembrane-protein; TMP: transmembrane protein; signal: signal peptide; Hyl, 2, 3, : hypervariable domains.

Figure 6: Direct repeats in the proteins of different AIDS virus isolates.

These examples are derived from the aligned sequences of gag (a, b), F (c,d) an env (e, f, g, h) shown in figure 3. The two elements of the direct repeat are boxed, while degenerated positions are underlined.

The invention thus pertains more specifically to the proteins, polypeptides or glycoproteins including the polypeptidic strucutres shown in the drawings. The first and last amino-acid residues of these proteins, polypeptides or glycoproteins carry numbers computed from a first aminoacid of the open-reading frames concerned, although these numbers do not correspond exactly to those of the LAV_{ELI} or LAV_{MAL} proteins concerned, rather to those of the LAVBRU corresponding proteins or sequences shown in figs. 3A, 3B and 3C. Thus a number corresponding to a "first amino-acid residue" of a LAV_{ELI} protein corresponds to the number of the first amino-acyl residue of the corresponding LAVBRU protein which, in any of figs. 3A, 3B or 3C is in direct alignment with the corresponding first amino-acid of the LAVELI protein. Thus the sequences concerned can be read from figs. 7A-7J and 8A-8I, to the extent where they do not appear with sufficient clarity from Figs. 3A-3F.

The preferred protein sequences of this invention extend from the corresponding "first" and "last" amino-acid residues (reference is also made to the protein(s)- or glycoprotein(s)-portions including part of the sequences which follow:

OMP or gp110 proteins, including precursors :

1 to 530

OMP or gp110 without precursor:

34-530

Sequence carrying the TMP or gp41 protein :

531-877, particularly

680-700

well conserved stretches of OMP:

37-130,

211-289 and

488-530

well conserved stretch found at the end of the OMP and the beginning of TMP:

490-620.

Proteins containing or consisting of the "well conserved stretches" are of particular interest for the production of immunogenic compositions and (preferably in relation to the stretches of the env protein) of vaccine compositions against the LAV-viruses of class 1 as above-defined.

The invention concerns more particularly all the DNA fragments which have been more specifically referred to in the drawings and which correspond to open reading frames. It will be understood that the man skilled in the art will be able to obtain them all, for instance by cleaving an entire DNA corresponding to the complete genome of either LAV_{ELI} or of LAV_{MAL}, such as by cleavage by a

partial or complet digestion thereof with a suitable restriction enzyme and by the subsequent recovery of the relevant fragments. The different DNAs disclosed above can be resorted to also as a source of suitable fragments. The techniques disclosed in PCT application for the isolation of the fragments which can then be included in suitable plasmids are applicable here too.

Of course other methods can be used. Some of them have been examplified in European Application Nr. 178,978 filed on September 17, 1985. Reference is for instance made to the following methods.

- a) DNA can be transfected into mammalian cells with appropriate selection markers by a variety of techniques, calcium phosphate precipitation, polyethylene glycol, protoplast-fusion, etc..
- b) DNA fragments corresponding to genes can be cloned into expression vectors for E. coli, yeast- or mammalian cells and the resultant proteins purified.
- c) The provival DNA can be "shot-gunned" (fragmented) into procaryotic expression vectors to generate fusion polypeptides. Recombinant producing antigenically competent fusion proteins can be identified by simply screening the recombinants with antibodies against LAV antigens.

The invention further refers more specifically to DNA recombinants, particularly modified vectors including any of the preceding DNA sequences and adapted to transform corresponding microorganisms or cells, particularly eucaryotic cells such as yeasts, for instance saccharomyces cerevisiae, or higher eucaryotic cells, particularly cells of mammals, and to permit expression of said DNA sequences in the corresponding microorganisms or cells. General methods of that type have been recalled in the abovesaid PCT international patent application PCT/EP 85/00548 filed on October 18, 1985.

More particularly the invention relates to such modified DNA recombinant vectors modified by the abovesaid DNA sequences and which are capable of transforming higher eucaryotic cells particularly mammalian cells. Preferably any of the abovesaid sequences are placed under the direct control of a promoter contained in said vectors and which is recognized by the polymerases of said cells, such that the first nucleotide codons expressed correspond to the first triplets of the above-defined ----DNA-sequences. Accordingly this invention also relates to the corresponding DNA fragments which can be obtained from genomas of LAV_{FII} or LAV MAL or corresponding cDNAs by any appropriate method. For instance such a method comprises cleaving said LAV genomas or cDNAs by restriction enzymes preferably at the level of restriction

sites surrounding said fragments and close to the opposite extremities respectively thereof, recovering and identifying the fragments sought according to sizes, if need be checking their restriction maps or nucleotide sequences (or by reaction with monoclonal antibodies specifically directed against epitopes carried by the polypeptides encoded by said DNA fragments), and further if need be, trimming the extremities of the fragments, for instance by an exonucleolytic enzyme such as Bal31, for the purpose of controlling the desired nucleotide-sequences of the extremities of said DNA fragments or, conversely, repairing said extremities with Klenow enzyme and possibly ligating the latter to synthetic polynucleotide fragments designed to permit the reconstitution of the nucleotide extremities of said fragments. Those fragments may then be inserted in any of said vectors for causing the expression of the corresponding polypeptide by the cell transformed therewith. The corresponding polypeptide can then be recovered from the transformed cells, if need be after lysis thereof, and purified, by methods such as electrophoresis. Needless to say that all conventional methods for performing these operations can be resorted to.

The invention also relates more specifically to cloned probes which can be made starting from any DNA fragment according to this invention, thus to recombinant DNAs containing such fragments, particularly any plasmids amplifiable in procaryotic or eucaryotic cells and carrying said fragments.

Using the cloned DNA fragments as a molecular hybridization probe - either by labelling with radio-nucleotides or with fluorescent reagents -LAV virion RNA may be detected directly in the blood, body fluids and blood products (e.g. of the antihemophylic factors such as Factor VIII concentrates) and vaccines, i.e. hepatitis B vaccine It has already been shown that whole virus can be detected in culture supernatants of LAV producing cells. A suitable method for achieving that detection comprises immobilizing virus onto a support, e.g. nitrocellulose filters, etc., disrupting the virion and hybridizing with labelled (radiolabelled or "cold" fluorescent- or enzyme-labelled) probes. Such an approach has already been developed for Hepatitis B virus in peripheral blood (according to SCOTTO J. et al. Hepatology (1983), 3, 379-384).

Probes according to the invention can also be used for rapid screening of genomic DNA derived from the tissue of patients with LAV related symptoms, to see if the proviral DNA or RNA present in host tissue and other tissues can be related to that of LAV_{ELI} or LAV_{MAL}.

A method which can be used for such screening comprises the following steps: extraction of DNA from tissue, restriction enzyme cleavage of said DNA, electrophoresis of the fragments and

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Southern blotting of genomic DNA from tissues, subsequent hybridization with labelled cloned LAV proviral DNA. Hybridization in situ can also be used.

Lymphatic fluids and tissues and other nonlymphatic tissues of humans, primates and other mammalian species can also be screened to see if other evolutionnary related retrovirus exist. The methods referred to hereabove can be used, although hybridization and washings would be done under non stringent conditions.

The DNAs or DNA fragments according to the invention can be used also for achieving the expression of viral antigens of LAV_{ELI} or LAV_{MAL} for diagnostic purposes.

The invention relates generally to the polypeptides themselves, whether synthetized chemically isolated from viral preparation or expressed by the different DNAs of the inventions, particularly by the ORFs or fragments thereof, in appropriate hosts, particularly procaryotic or eucaryotic hosts, after transformation thereof with a suitable vector previously modified by the corresponding DNAs.

More generally, the invention also relates to any of the polypeptide fragments (or molecules, particularly glycoproteins having the same polypeptidic backbone as the polypeptides mentioned hereabove) bearing an epitope characteristic of a protein or glycoprotein of LAV_{ELI} or LAV_{MAL}, which polypeptide or molecule then has N-terminal and C-terminal extremities respectively either free or, independently from each other, covalently bond to aminoacids other than those which are normally associated with them in the larger polypeptides or glycoproteins of the LAV virus, which last mentioned aminoacids are then free or belong to another polypeptidic sequence. Particularly the invention relates to hybrid polypeptides containing any of the epitope-bearing-polypeptides which have been defined more specifically hereabove, recombined with other polypeptides fragments normally foreign to the LAV proteins, having sizes sufficient to provide for an increased immunogenicity of the epitope-bearing-polypeptide yet, said foreign polypeptide fragments either being immunogenically inert or not interfering with the immunogenic properties of the epitope-bearing-polypeptide.

Such hybrid polypeptides which may contain from 5 up to 150, even 250 aminoacids usually consist of the expression products of a vector which contained ab initio a nucleic acid sequence expressible under the control of a suitable promoter or replicon in a suitable host, which nucleic acid sequence had however beforehand been modified by insertion therein of a DNA sequence encoding said epitope-bearing-polypeptide.

Said epitope-bearing-polypeptides, particularly those whose N-terminal and C-terminal aminoacids

are free, are also accessible by chemical synthesis, according to technics well known in the chemistry of proteins.

The synthesis of peptides in homogeneous solution and in solid phase is well known.

In this respect, recourse may be had to the method of synthesis in homogenous solution described by Houbenweyl in the work entitled "Methoden der Organischen Chemie" (Methods of Organic Chemistry) edited by E. WUNSCH., vol. 15-I and II, THIEME, Stuttgart 1974.

This method of synthesis consists of successively condensing either the successive aminoacids in twos, in the appropriate order or successive peptide fragments previously available or formed and containing already several aminoacyl residues in the appropriate order respectively. Except for the carboxyl and amino-groups which will be engaged in the formation of the peptide bonds, care must be taken to protect beforehand all other reactive groups borne by these aminoacyl groups or fragments. However, prior to the formation of the peptide bonds, the carboxyl groups are advantageously activated, according to methods well known in the synthesis of peptides. Alternatively, recourse may be had to coupling reactions bringing into play conventional coupling reagents, for instance of the carbodiimide type, such as 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. When the aminoacid group used carries an additional amine group (e.g. lysine) or another acid function (e.g. glutamic acid). these groups may be protected by carbobenzoxy or t-butyloxycarbonyl groups, as regards the amine groups, or by t-butylester groups, as regards the carboxylic groups. Similar procedures are available for the protection of other reactive groups, for example, SH group (e.g. in cysteine) can be protected by an acetamidomethyl or paramethoxybenzyl group.

In the case of progressive synthesis, aminoacid by aminoacid, the synthesis starts preferably by the condensation of the C-terminal aminoacid with the aminoacid which corresponds to the neighboring aminoacyl group in the desired sequence and so on, step by step, up to the N-terminal aminoacid. Another preferred technique can be relied upon is that described by R.D. Merrifield in "solid phase peptide synthesis" (J. Am. Chem. Soc., 45, 2149-2154).

In accordance with the Merrifield process, the first C-terminal aminoacid of the chain is fixed to a suitable porous polymeric resin, by means of its carboxylic group, the amino group of said aminoacid then being protected, for example by a t-butyloxycarbonyl group.

When the first C-terminal aminoacid is thus fixed to the resin, the protective group of the amine group is removed by washing the resin with an

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acid, i.e. trifluoroacetic acid, when the protective group of the amine group is a t-butyloxycarbonyl group.

Then the carboxylic group of the second aminoacid which is to provide the second aminoacyl group of the desired peptidic sequence, is coupled to the deprotected amine group of the C-terminal aminoacid fixed to the resin. Preferably, the carboxyl group of this second aminoacid has been activated, for example by dicyclohexyl-carbodiimide, while its amine group has been protected, for example by a t-butyloxycarbonyl group. The first part of the desired peptide chain, which comprising the first two aminoacids, is thus obtained. As previously, the amine group is then deprotected, and one can further proceed with the fixing of the next aminoacyl group and so forth until the whole peptide sought is obtained.

The protective groups of the different side groups, if any, of the peptide chain so formed can then be removed. The peptide sought can then be detached from the resin, for example, by means of hydrofluoric acid, and finally recovered in pure form from the acid solution according to conventional procedures.

As regards the peptide sequences of smallest size and bearing an epitope or immunogenic determinant, and more particularly those which are readily accessible by chemical synthesis, it may be required, in order to increase their in vivo immunogenic character, to couple or "conjugate" them covalently to a physiologically acceptable and non toxic carrier molecule.

By way of examples of carrier molecules or macromolecular supports which can be used for making the conjugates according to the invention, will be mentioned natural proteins, such as tetanic toxoid, ovalbumin, serum-albumins, hemocyanins, etc.. Synthetic macromolecular carriers, for example polysines or poly(D-L-alanine)-poly(L-lysine)s, can be used too.

Other types of macromolecular carriers which can be used, which generally have molecular weights higher than 20,000, are known from the literature.

The conjugates can be synthesized by known processes, such as described by Frantz and Robertson in "Infect. and Immunity", 33, 193-198 (1981), or by P.E. Kauffman in "Applied and Environmental Microbiology", October 1981 Vol. 42, nº 4, 611-614.

For instance the following coupling agents can be used: glutaric aldehyde, ethyl chloroformate, water-soluble carbodiimides (N-ethyl-N'(3-dimethylaminopropyl) carbodiimide, HC1), diisocyanates, bis-diazobenzidine, di- and trichloros-triazines, cyanogen bromides, benzaquinone, as well as coupling agents mentioned in "Scand. J.

Immunol., 1978, vol. 8, p. 7-23 (Avrameas, Ternynck, Guesdon).

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Any coupling process can be used for bonding one or several reactive groups of the peptide, on the one hand, and one or several reactive groups of the carrier, on the other hand. Again coupling is advantageously achieved between carboxyl and amine groups carried by the peptide and the carrier or vice-versa in the presence of a coupling agent of the type used in protein synthesis, i.e. 1ethyl-3-(3-dimethylaminopropyl)-carbodiimide. hydroxybenzotriazole, etc.. Coupling between amine groups respectively borne by the peptide and the carrier can also be made with glutaraldehyde, for instance, according to the method described by BOQUET, P. et al. (1982) Molec. Immunol., 19, 1441-1549, when the carrier is hemocyanin.

The immunogenicity of epitope-bearing-peptides can also be reinforced, by oligomerisation thereof, for example in the presence of glutaral-dehyde or any other suitable coupling agent. In particular, the invention relates to the water soluble immunogenic oligomers thus obtained, comprising particularly from 2 to 10 monomer units.

The glycoproteins, proteins and polypeptides (generally designated hereafter as "antigens" of this invention, whether obtained (by methods such as disclosed in the earlier patent applications referred to above) in a purified state from LAV_{ELI} or LAV_{MAL} virus preparations or - as concerns more particularly the peptides - by chemical synthesis, are useful in processes for the detection of the presence of anti-LAV antibodies in biological media, particularly biological fluids such as sera from man or animal, particularly with a view of possibly diagnosing LAS or AIDS.

Particularly the invention relates to an in vitro process of diagnosis making use of an envelope glycoprotein (or of a polypeptide bearing an epitope of this glycoprotein of LAV_{ELI} or LAV_{MAL} for the detection of anti-LAV antibodies in the serums of persons who carry them. Other polypeptides particular those carrying an epitope of a core protein - can be used too.

A preferred embodiment of the process of the invention comprises :

- depositing a predetermined amount of one or several of said antigens in the cups of a titration microplate;
- introducing of increasing dilutions of the biological fluid, i.e. serum to be diagnosed into these cups;
- incubating the microplate;
- washing carefully the microplate with an appropriate buffer;
- adding into the cups specific labelled antibodies directed against blood im-

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munoglobulins and

detecting the antigen-antibody-complex formed, which is then indicative of the presence of LAV antibodies in the biological fluid.

Advantageously the labelling of the antiim-munoglobulin antibodies is achieved by an enzyme selected from among those which are capable of hydrolysing a substrate, which substrate undergoes a modification of its radiation-absorption, at least within a predetermined band of wavelengths. The detection of the substrate, preferably comparatively with respect to a control, then provides a measurement of the potential risks or of the effective presence of the disease.

Thus preferred methods immuno-enzymatic or also immunofluorescent detections, in particular according to the ELISA technique. Titrations may be determinations by immunofluorescence or direct or indirect immuno-enzymatic determinations. Quantitative titrations of antibodies on the serums studied can be made.

The invention also relates to the diagnostic kits themselves for the in vitro detection of antibodies against the LAV virus, which kits comprise any of the polypeptides identified herein, and all the biological and chemical reagents, as well as equipment, necessary for peforming diagnostic assays. Preferred kits comprise all reagents required for carrying out ELISA assays. Thus preferred kits will include, in addition to any of said polypeptides, suitable buffers and anti-human immunoglobulins, which anti-human immunoglobulins are labelled either by an immunofluorescent molecule or by an enzyme. In the last instance preferred kits then also comprise a substrate hydrolysable by the enzyme and providing a signal, particularly modified absorption of a radiation, at least in a determined wavelength, which signal is then indicative of the presence of antibody in the biological fluid to be assayed with said kit.

It can of course be of advantage to use several proteins or polypeptides not only of both LAV_{ELI} and LAV_{MAL}, but also of any or both of them together with homologous proteins or polypeptides of earlier described viruses, e.g. of LAV_{BRU} or HTLV_{III} or ARV, etc..

The invention also relates to vaccine compositions whose active principle is to be constituted by any of the antigen, i.e. the hereabove disclosed polypeptides whole antigens, of either LAV_{ELI} or LAV_{MAL}, or both, particularly the purified gp110 or immunogenic fragments thereof, fusion polypeptides or oligopeptides in association with a suitable pharmaceutical or physiologically acceptable carrier.

A first type of preferred active principle is the gp110 immunogen of said immunogens.

Other preferred active principles to be consid-

ered in that fields consist of the peptides containing less than 250 aminoacid units, preferably less than 150, particularly from 5 to 150 aminocid residues, as deducible for the complete genomas of LAV_{ELI} and LAV_{MAL} and even more preferably those peptides which contain one or more groups selected from Asn-X-Ser and Asn-X-Ser as defined above. Preferred peptides for use in the production of vaccinating principles are peptides (a) to (f) as defined above. By way of example having no limitative character, there may be mentioned that suitable dosages of the vaccine compositions are those which are effective to elicit antibodies in vivo, in the host, particularly a human host. Suitable doses range from 10 to 500 micrograms of polypeptide, protein or glycoprotein per kg, for instance 50 to 100 micrograms per kg.

The different peptides according to this invention can also be used themselves for the production of antibodies, preferably monoclonal antibodies specific of the different peptides respectively. For the production of hybridomas secreting said monoclonal antibodies, conventional production and screening methods are used. These monoclonal antibodies, which themselves are part of the invention then provide very useful tools for the identification and even determination of relative proportions of the different polypeptides or proteins in biological samples, particularly human samples containing LAV or related viruses.

The invention further relates to the hosts (procaryotic or eucaryotic cells) which are transformed by the above mentioned recombinants and which are capable of expressing said DNA fragments.

Finally the invention also concerns vectors for the transformation fo eucaryotic cells of human origin, particularly lymphocytes, the polymerase of which are capable of recognizing the LTRs of LAV. Particularly said vectors are characterized by the presence of a LAV LTR therein, said LTR being then active as a promoter enabling the efficient transcription and translation in a suitable host of a DNA insert coding for a determined protein placed under its controls.

It must be understood that the claims which follow are also intended to cover all equivalents of the products (glycoproteins, polypeptides, DNAs, etc...) whereby an equivalent is a product, i.e. a polypeptide which may distinguish from a determined one defined in any of said claims, say through one or several amino-acids, while still having substantially the same immunological or immunogenic properties. A similar rule of equivalency shall apply to the DNAs, it being understood that the rule of equivalency will then be tied to the rule of equivalency pertaining to the polypeptides which they encode.

It will also be understood that all the literature referred to hereinbefore or hereinafter, and all patent applications or patents not specifically identified herein but which form counterparts of those specifically designated herein must be considered as incorporated herein by reference.

It should further be mentioned that the invention further relates to immunogenic compositions containing preferably not only any of the polypeptides more specifically identified above and which have the aminoacid-sequences of LAV $_{\rm ELI}$ and LAV $_{\rm MAL}$ which have been identified, but corresponding peptidic sequences to previously defined LAV proteins too.

In that respect the invention relates more particularly to the particular polypeptides which have the sequences corresponding more specifically to the LAV_{BRU} sequences which have been referred to earlier, i.e. the sequences extending between the following first and last aminoacids, of the LAV_{BRU} proteins themselves, i.e. the polypeptides having sequences contained in the LAV_{BRU} OMP or LAV_{BRU} TMP or sequences extending over both, particularly those extending from between the following positions of the aminoacids included in the env open reading frame of the LAV_{BRU} genome,

1-530 34-530 and more preferably 531-877, particularly 680-700 37-130 211-289 488-530

490-620.

These different sequences can be used for any of the above defined purposes and in any of the compositions which have been disclosed.

Finally the invention also relates to the different antibodies which can be formed specifically against the different peptides which have been disclosed herein, particularly to the monoclonal antibodies which recognize them specifically. The corresponding hybridomas which can be formed starting from spleen cells previously immunized with such peptides which are fused with appropriate myeloma cells and selected according to standard procedures also form part of the invention.

Phage λ clone E-H12 derived from LAV_{ELI} infected cells has been deposited at the "Collection Nationale des Cultures de Micro-organismes" (National Collection of Cultures of Microorganisms) (CNCM) of the Pasteur Institute of Paris France, under no I-550 on May 9th, 1986.

Phage λ clone M-H11 derived from LAV_{MAL} infected cells has been deposited at the CNCM under no I-551 on May 9th, 1986.

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Claims

- The virus LAV_{EU} whose RNA corresponds to the cDNA of figs. 7A-7J.
- The virus LAV_{MAL} whose RNA corresponds to the cDNA of figs. 8A-8I.
- 3. The DNA, such as cDNA, of figs. 7A-7J.
- 4. The DNA, such as cDNA, of figs. 8A-8I.
- A DNA recombinant containing the DNA of claim 3 or 4.
- **6.** A probe containing a cloned nucleic acid of anyone of claims 3 to 5.
- 7. A method for identifying the presence or absence in a host tissue of LAV_{ELI} or LAV_{MAL} or a provirus thereof which comprises hybridizing DNA obtained from said tissue with a probe of claim 6 and detecting the presence or absence of said virus or provirus in said tissue depending upon whether or not there is hybridization with said probe.
- 8. A protein or glycoprotein which is encoded by an open reading frame of the DNA of claim 3 or 4 or a part of the protein or glycoprotein which corresponds to the stretch extending from aminoacyl residue 37 to aminoacyl residue 130, or from aminoacyl residue 211 to aminoacyl residue 289, or from aminoacyl residue 488 to aminoacyl residue 530 of fig. 3.
- A part of the protein or glycoprotein of claim 8 which corresponds to the stretch extending from the aminoacyl residue 490 to the aminoacyl residue 620 of fig. 3.
- 10. A part of the protein or glycoprotein of claim 8 whose amino acid sequence consists essentially of all or part of the sequences which follow:

OMP or gp110 proteins, including precursors: 1 to 530

OMP or gp110 without precursor: 34-530

Sequence carrying the TMP or gp41 protein:

531-877, particularly 😙

680-700

well conserved stretches of OMP:

37-130,

211-289 and

488-530

or well conserved stretch found at the end of the OMP and the beginning of TMP: 490-620.

- 11. A method for the in vitro detection of the presence of antibodies directed against LAV_{ELI} or LAV_{MAL} in a human body fluid which comprises: contacting said body fluid with antigens obtained from the viruses of claim 1 or 2 or consisting of a protein, glycoprotein or part thereof of any one of claims 8 to 10; and detecting the immunological reaction between said antigens and said antibodies.
- 15 12. The method of claim 11 which comprises the steps of:
 - depositing a predetermined amount of one or several of said antigens in the cups of a titration microplate;
 - introducing increasing dilutions of the biological fluid, i.e., serum, to be diagnosed into these cups;
 - incubating the microplate;
 - washing carefully the microplate with an appropriate buffer;
 - adding into the cups specific labelled antibodies directed against blood immunoglobulins; and
 - detecting the antigen-antibody complex formed, which is then indicative of the presence of said antibodies in the biological fluid.
 - 13. A diagnostic kit for the in vitro detection of antibodies against the virus of claim 1 or 2 or both, which contains an antigen obtained from said virus or consisting of a protein, glycoprotein or part thereof of any of claims 8 to 10, and the biological and chemical reagents, as well as equipment, necessary for performing diagnostic assays.
 - 14. An immunogenic composition containing an antigen of the virus of claim 1 or 2 or any immunogenic protein, glycoprotein or part thereof of any of claims 8 to 10 in association with a pharmaceutically and/or physiologically acceptable carrier.
- 50 **15.** The immunogenic composition of claim 14 wherein the immunogenic glycoprotein or part thereof is the gp110 envelope glycoprotein or part thereof.
 - 16. The immunogenic composition of claim 14 which contains the part of the protein or glycoprotein of claim 10.

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- 17. The antibodies, particularly monoclonal antibodies, formed against any of the proteins, glycoproteins or parts thereof of any of claims 8 to 10.
- The cells transformed with the DNA recombinant of claim 5.
- 19. A process for making the DNA of claim 3 or 4, comprising the step(s) of:
 - isolating the DNA of LAV_{ELI} or LAV_{MAL}; or
 - cleaving the DNA of LAV_{ELI} or LAV_{MAL} with a suitable restriction enzyme and subsequently recovering the parts thereof; or
 - isolating the DNA from cells transformed with the DNA of LAV_{ELI} or LAV_{MAL} in accordance with European patent application 178 978 of September 17, 1985.
- 20. A process for making the protein, glycoprotein or part thereof of any of claims 8 to 10 comprising the step(s) of:
 - expressing the DNA of claim 19 in a cell transformed therewith and then recovering the protein, peptide or part thereof; or
 - synthesizing the protein, peptide or part thereof in homogeneous solution by successively condensing successive amino acids or successive peptide fragments in an appropriate order.

Patentansprüche

- LAV_{ELI}-Virus, dessen RNA der cDNS der Fig.7A-7J entspricht.
- LAV_{MAL}-Virus, dessen RNA der cDNS der Fig.8A-8I entspricht.
- 3. DNS sowie cDNS der Fig.7A-7J.
- 4. DNS sowie cDNS der Fig.8A-8I.
- Rekombinante DNS, enthaltend DNS nach Anspruch 3 oder 4.
- Sonde, enthaltend eine geklonte Nukleinsäure nach einem der Ansprüche 3 bis 5.
- 7. Verfahren zur Feststellung der An- oder Abwesenheit von LAV_{ELI} oder LAV_{MAL} oder eines Provirus davon in einem Gast-Gewebe, welches die Hybridisierung der aus dem Gewebe erhaltenen DNS mit einer Sonde des Anspruchs 6 und die Feststellung der An- oder Abwesenheit des Virus oder Provirus in dem Gewebe in Abhängigkeit davon, ob eine Hybri-

disierung mit der Sonde stattfindet oder nicht, umfaßt.

- 8. Protein oder Glycoprotein, welches durch ein offenes Ableseraster der DNS von Anspruch 3 oder 4 codiert wird, oder ein Teil eines Proteins oder Glycoproteins, das einem Abschnitt entspricht, der sich vom Aminoacylrest 37 bis Aminoacylrest 130 oder vom Aminoacylrest 211 bis Aminoacylrest 289 oder vom Aminoacylrest 488 bis Aminoacylrest 530 der Fig.3 erstreckt.
- Protein- oder Glycoproteinteil nach Anspruch
 das einem Abschnitt entspricht, der sich vom Aminoacylrest 490 bis zum Aminoacylrest
 der Fig.3 erstreckt.
- Protein- oder Glycoproteinteil nach Anspruch 8, dessen Aminosäurensequenz sich im wesentlichen aus allen oder einem Teil der folgenden Sequenzen zusammensetzt: OMP oder gp110-Proteine einschließlich der Vorstufen:

1 bis 530

OMP oder gp110 ohne Vorstufen:

34 bis 530

Sequenz, die TMP- oder gp41-Protein trägt:

531 bis 877, insbesondere

680 bis 700

gut erhaltene Abschnitte von OMP:

37 bis 130,

211 bis 289 und

488 bis 530

oder gut erhaltene Abschnitte, die sich am Ende des OMP's oder am Anfang des TMP's finden:

490 bis 620.

- 11. Verfahren zur in vitro-Bestimmung der Anwesenheit von Antikörpern gegen LAV_{ELI} oder LAV_{MAL} in menschlichen Körperflüssigkeiten die umfaßt: Kontaktierung der Körperflüssigkeit mit Antigenen, die aus den Viren nach Anspruch 1 oder 2 erhalten wurden, oder aus Proteinen, Glycoproteinen oder Teilen davon nach einem der Ansprüche 8 bis 10 bestehen, und Erfassung der immunologischen Reaktion zwischen Antigenen und Antikörpern.
 - 12. Verfahren nach Anspruch 11, das die folgenden Schritte umfaßt:
 - Einbringen einer vorbestimmten Menge eines oder mehrerer der genannten Antigene in die Näpfchen einer Microtiterplatte;
 - Zugabe ansteigender Verdünnungen der biologischen Flüssigkeit, beispielsweise

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Serum, das in den Näpfchen untersucht werden soll;

- Inkubierung der Microplatte:
- vorsichtiges Waschen der Microplatte mit einem geeigneten Puffer;
- Hinzufügung spezifischer markierter Antikörper gegen Blutimmunoglobuline in die Näpfchen und
- Erfassung des gebildeten Antigen-Antikörperkomplexes, welcher einen Hinweis auf die Anwesenheit besagter Antikörper in der biologischen Flüssigkeit ist.
- 13. Diagnostische Ausstattung zur in vitro-Bestimmung von Antikörpern gegen die Viren nach Anspruch 1 und/oder 2, die ein Antigen enthält, das aus besagtem Virus erhalten wurde oder das aus einem Protein, Glycoprotein oder Teil davon nach einem der Ansprüche 8 bis 10 besteht, und die biologischen und chemischen Reagentien sowie Ausrüstungen, die zur Durchführung des diagnostischen Tests erforderlich sind.
- 14. Immunogene Zusammensetzung, enthaltend ein Antigen des Virus nach Anspruch 1 oder 2, oder irgendein immunogenes Protein, Glycoprotein oder Teil davon nach einem der Ansprüche 8 bis 10 in Verbindung mit einem pharmazeutisch und/oder physiologisch akzeptablem Träger.
- 15. Immunogene Zusammensetzung nach Anspruch 14, worin das immunogene Glycoprotein oder Teil davon das gp110 Hüll-Glycoprotein oder ein Teil davon ist.
- Immunogene Zusammensetzung nach Anspruch 14, die ein Teil des Proteins oder Glycoproteins nach Anspruch 10 enthält.
- Antikörper, insbesondere monoklonale Antikörper, die gegen irgendeines der Proteine, Glycoproteine oder Teile davon nach einem der Ansprüche 8 bis 10 gebildet wurden.
- Transformierte Zellen mit rekombinanter DNS nach Anspruch 5.
- 19. Verfahren zur Herstellung der DNS nach Anspruch 3 oder 4, umfassend den Schritt/die Schritte:
 - Isolierung der DNS von LAV_{ELI} oder LAV_{MAL} oder
 - Spaltung der DNS von LAV_{ELI} oder LAV_{MAL} mit einem geeigneten Restriktionsenzym und anschließende Gewinnung der erhaltenen Teile oder

- Isolierung der DNS aus Zellen, die mit der DNS von LAV_{ELI} oder LAV_{MAL} entsprechend der europäischen Patentanmeldung 178 978 vom 17.September 1985 transformiert wurden.
- 20. Verfahren zur Herstellung des Proteins, Glycoproteins oder Teilen davon nach einem der Ansprüche 8 bis 10, umfassend den Schritt/die Schritte:
 - Expression der DNS nach Anspruch 19 in einer damit transformierten Zelle und Gewinnung des Proteins, Peptids oder eines Teiles davon oder
 - Synthese der Proteine, Peptide oder Teile davon in einer homogenen Lösung durch aufeinanderfolgende Kondensation der aufeinanderfolgenden Aminosäuren oder Peptidfragmente in einer geeigneten Reihenfolge.

Revendications

- Le virus LAV_{ELI} dont l'ARN correspond à l'ADNc des Figures 7A-7J.
- Le virus LAV_{MAL} dont l'ARN correspond à l'ADNc des Figures 8A-8I.
- 30 3. L'ADN, tel que l'ADNc, des Figures 7A-7J.
 - 4. L'ADN, tel que l'ADNc, des Figures 8A-8I.
 - 5. ADN recombinant contenant l'ADN tel que défini à l'une des revendications 3 et 4.
 - Sonde contenant un acide nucléique cloné tel que défini à l'une quelconque des revendications 3 à 5.
 - 7. Procédé pour identifier la présence ou l'absence, dans un tissu hôte, du LAV_{EU} ou LAV_{MAL}, ou d'un provirus de ceux-ci, qui comprend :
 - l'hybridation de l'ADN obtenu à partir dudit tissu avec une sonde telle que définie à la revendication 6; et
 - la détection de la présence ou de l'absence dudit virus ou dudit provirus dans ledit tissu selon qu'il y ait ou non hybridation avec ladite sonde.
 - 8. Protéine ou glycoprotéine qui est codée par un cadre de lecture ouvert de l'ADN tel que défini à l'une des revendications 3 et 4, ou partie de la protéine ou glycoprotéine qui correspond au segment s'étendant à partir du résidu aminoacyle 37 au résidu aminoacyle 130, ou du résidu aminoacyle 211 au résidu aminoacyle 289,

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ou du résidu aminoacyle 488 au résidu aminoacyle 530 de la Figure 3.

- 9. Partie de la protéine ou glycoprotéine selon la revendication 8, qui correspond au segment s'étendant à partir du résidu aminoacyle 490 au résidu aminoacyle 620 de la Figure 3.
- 10. Partie de la protéine ou glycoprotéine selon la revendication 8, dont la séquence d'acides aminés consiste essentiellement en la totalité ou une partie des séquences qui suivent :
 - protéines OMP ou gp110, incluant les précurseurs :

1 à 530

- OMP ou gp110 sans précurseur : 34-530
- séquence portant la protéine TMP ou gp41 :

531-877, en particulier 680-700

 les segments bien conservés d'OMP : 37-130,

211-289 et

488-530; ou

 le segment bien conservé que l'on trouve à l'extrémité de l'OMP et au début de la TMP :

490-620.

- 11. Procédé pour la détection in vitro de la présence d'anticorps dirigés contre LAV_{ELI} ou LAV_{MAL}, dans un fluide corporel humain, qui comprend :
 - la mise en contact dudit fluide corporel avec des antigènes obtenus à partir des virus tels que définis à l'une des revendications 1 et 2, ou consistant en une protéine, glycoprotéine, ou partie de celles-ci, telle que définie à l'une quelconque des revendications 8 à 10; et
 - la détection de la réaction immunologique entre lesdits antigènes et lesdits anticorps.
- **12.** Procédé selon la revendication 11, qui comprend les étapes consistant à :
 - déposer une quantité prédéterminée de l'un ou plusieurs desdits antigènes dans les cuvettes d'une plaque de microtitration :
 - introduire dans ces cuvettes des dilutions
 croissantes du fluide biologique, à savoir,
 du sérum, à diagnostiquer;
 - faire incuber la microplaque ;
 - laver soigneusement la microplaque avec un tampon approprié;
 - ajouter dans les cuvettes des anticorps

- marqués spécifiques, dirigés contre les immunoglobulines du sang ; et
- détecter le complexe antigène anticorps formé, qui dénote alors la présence desdits anticorps dans le fluide biologique.
- 13. Kit de diagnostic pour la détection in vitro d'anticorps dirigés contre le virus tel que défini à l'une des revendications 1 et 2 ou aux deux, qui contient un antigène obtenu à partir dudit virus ou consistant en une protéine, glycoprotéine, ou partie de celles-ci, telle que définie à l'une quelconque des revendications 8 à 10, et les réactifs biologiques et chimiques, ainsi que l'équipement, nécessaires pour effectuer des essais diagnostiques.
- 14. Composition immunogène contenant un antigène du virus tel que défini à l'une des revendications 1 et 2, ou n'importe quelle protéine, glycoprotéine ou partie de celles-ci, immunogène, telle que définie à l'une quelconque des revendications 8 à 10, en association avec un support pharmaceutiquement et/ou physiologiquement acceptable.
- 15. Composition immunogène selon la revendication 14, dans laquelle la glycoprotéine ou partie de celle-ci, immunogène, est la glycoprotéine d'enveloppe gp110 ou une partie de celleci.
- 16. Composition immunogène selon la revendication 14, qui contient la partie de la protéine ou glycoprotéine telle que définie à la revendication 10.
- 17. Anticorps, particulièrement anticorps monoclonaux, formés contre l'une quelconque des protéines, glycoprotéines, ou parties de celles-ci, telles que définies à l'une quelconque des revendications 8 à 10.
- 18. Cellules transformées par l'ADN recombinant tel que défini à la revendication 5.
- 19. Procédé de préparation de l'ADN tel que défini à l'une des revendications 3 et 4, comprenant la (ou les) étape(s) consistant à :
 - isoler l'ADN du LAVELI ou LAVMAL; ou
 - cliver l'ADN du LAV_{ELI} ou LAV_{MAL} avec une enzyme de restriction appropriée et, par la suite, récupérer les parties de celui-ci; ou
 - isoler l'ADN à partir de cellules transformées par l'ADN du LAV_{ELI} ou LAV_{MAL}, conformément à la demande de brevet européen nº 178 978 du 17 septembre

1985.

- 20. Procédé de fabrication de la protéine, glycoprotéine ou partie de celles-ci, telle que définie à l'une quelconque des revendications 8 à 10, comprenant la (ou les) étape(s) consistant à :
 - exprimer l'ADN tel que défini à la revendication 19 dans une cellule transformée par celui-ci, puis récupérer la protéine, le peptide, ou une partie de ceux-ci; ou synthétiser la protéine, le peptide, ou une partie de ceux-ci, en solution homogène, par condensation successive des acides aminés successifs ou des fragments peptidiques successifs dans un ordre approprié.

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CACTTAACCCTGGCCTTTTAGAAACAGGAGA CONSTRAINTEULYSTHrGlySerGluGluI CAATCAACTCTCAAGACAGGATCAGAAGAAA CEUTYrCysValHisGlnArgIleAspValL CTCTATTGTGTACATCAAAGGATAGATGTAA COUTYCYSValHisGlnArgIleAspValL CTCTATTGTGTACATCAAAGGATAGATGTAA CALAALAALAThrLysAsnSerSerSerValS CAGGGAAATACAAAAAAAAAAGACCAGCAAGTGTCA AlaAlaAlaThrLysAsnSerSerSerValS GCAGCTGCCACAAAAAAACAGCAGCCAAAAAAA AlaAlaAlaThrLysAsnSerSerSerValS GCAGCTGCCACAAAAAAACAGCAGCCAATATCAC GlnGlyGlnMetIleHisGlnAlaIleSerP CAAAGGGCAAATGATACATCAGGCCATATCAC BOO IleGluGluLysAlaPheSerProGluValI ATAGAAGAAAAAGGCTTTCAGCCCAGAAGTGAA AlaThrProGlnAspLeuAsnHetHetLeuA GCCACCCCCACAAGATTTAAATATGATGCTGAA CAAATGTTAAAAAGATACCATCAATGAGGAAGA CAAATGTTAAAAAGATACCATCAATGAGGAAGAAGAACAAAAAAAA	TAAGCCTCAATAAAGCTTGCCTTGAGTGCCTC CTCTGGTAACTAGAGATCCCTCAGACCACTCT GCCCGAACAGGGACTTTAAAGTGAAAGTAACA 200 GTTCTCTCGACGCAGGACTCGGCTTGCTGAGG CTGGTGAGTACGCCAGTTTTTGACTAGCGGAG CTGGTGAGTACGCCAATTTTTGACTAGCGGAG CTGGTGAGTACGCCAATTTTTGACTAGCGGAG CASSETVAILEUSETGLYGLYLYSLEUASPALA CCGTCAGTATTAAGCGGGGGAAAATTAGATGCA CLYLYSLYSLYSTYTATGLEULYSHISLEUVAI CGAAAGAAAAAATATAGACTGAAACATTTAGTA CASSETTATLEULYSTHTGLYSHISLEUVAI CACTTAACCCTGGCCTTTTAGAAACAGTAGAA CAACTCTCAAGACAGGATCAGAAGAAATT CUTYTCYSVALHISGLAATGLYSETGLUGLULLE CTTATTGTGTACATCAAAGGATAGATGTAAAA CSUTYTCYSVALHISGLAATGILEASPVALLYS CTCTATTGTGTACATCAAAGGATAGATGTAAAA CSUTYTCYSVALHISGLAATGILEASPVALLYS CTCTATTGTGTACATCAAAGGATAGATGTAAAA CSUTYTCYSVALHISGLAATGILEASPVALLYS CTCTATTGTGTACATCAAAGGATAGATGTAAAA CSUTYTCYSVALHISGLAATGILEASPVALLYS CTCTATTGTGTACATCAAAGGATAGATGTAAAA CSUTYTCYSVALHISGLAATGILEASPVALLYS CTCTATTGTGTACATCAAAGGATAGATGTAAAA CSUTYTCYSVALHISGLAATGAGGCCAAAAGACA ALAALAALATATAGAGCCAGCCATATCACCT CAAAGGGCAAAATGATACATCAGGCCCATATCACCT 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CCUTYTCYSVALHISGLAATGLEASPVALLYSAS CTCTATTGTGTACATCAAAGGATAGATGTAAAAGA CLUGLUILEGLAASALYSSETATGCILYSTHTGL GAAGGAAATACAAAATAAGAGCAGCCAAAAGACACA ALAALAALATTLYSASNSETSETSETVALSETGL GAAGGGCAAATACAAAATAAGAGCAGCAAGTGTCAGTCA GCAGCTGCCCACAAAAAAAACAGCAGCAGTGTCAGTCA GLAGLUGLULYSALSPHOSETPTOGLUVALLLEPT ATAGAAGAAAAAGGCTTTCAGCCCAGAAGTGATACC ALATATPTOGLAASPLEUASHNETNETBLEUASHIL GCCACCCCCACAAGATTTAAATATGATGCTGGAACAT CAAAATGTTAAAAAGATACCATCAATGAGGAAAGCTGCCCACAAATGATACCATTCACCTTGAACAT CAAAATGTTAAAAAGATACCATCAATGAGGAAAGCTGCCCACAAATGTTAAAAAGATACCATCAATGAGGAAAGCTGCCCACAAATGATACCATCAATGAGGAAAGCTGCCCACAAATGATACCATCAATGAGGAAAGCTGCCCACAAATGATACCATCAATGAGGAAAGCTGCCCACAAATGATACCATCAATGAGGAAAGCTGCCCACAAATGATACCATCAATGAGGAAAGCTGCCCACAAATGATGAAGAAACCATCAATGAAGGAAAGCTGCCCACAAATGATAAAAAGATACCATCAATGAGGGAAAGCTGCCCACAAAATGCTTAAAAAGATACCATCAATGAGGGAAAGCTGCCCACAAATGATAAAAAAAA	TAAGCCTCAATAAAGCTTGCCTTGAGTGCCTCAAGCC CTCTGGTAACTAGAGATCCCTCAGACCACTCTAGAC CCCGAACAGGGACTTTAAAGTGAAAGTAACAGGGA 200 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